

Cullin-3 Regulates Vascular Smooth Muscle Function and Arterial Blood Pressure via PPAR γ and RhoA/Rho-Kinase

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SUMMARY

Dominant-negative (DN) mutations in the nuclear hormone receptor peroxisome proliferator-activated receptor- γ (PPAR γ) cause hypertension by an unknown mechanism. Hypertension and vascular dysfunction are recapitulated by expression of DN PPAR γ specifically in vascular smooth muscle of transgenic mice. DN PPAR γ increases RhoA and Rho-kinase activity, and inhibition of Rho-kinase restores normal reactivity and reduces arterial pressure. RhoBTB1, a component of the Cullin-3 RING E3 ubiquitin ligase complex, is a PPAR γ target gene. Decreased RhoBTB1, Cullin-3, and neddylated Cullin-3 correlated with increased levels of the Cullin-3 substrate RhoA. Knockdown of Cullin-3 or inhibition of cullin-RING ligase activity in aortic smooth muscle cells increased RhoA. Cullin-RING ligase inhibition enhanced agonist-mediated contraction in aortic rings from normal mice by a Rho-kinase-dependent mechanism, and it increased arterial pressure in vivo. We conclude that Cullin-3 regulates vascular function and arterial pressure, thus providing a mechanistic link between mutations in Cullin-3 and hypertension in humans.

INTRODUCTION

PPAR γ is activated by thiazolidinediones (TZDs), a class of insulin-sensitizing drugs. TZD treatment attenuates the development of hypertension and inhibits atherosclerosis in type II diabetes patients (Halabi and Sigmund, 2005). Known adverse effects of TZD treatment include weight gain and systemic edema. Clinical studies report an increased risk of myocardial infarction and heart failure in patients taking the TZD rosiglitazone (Graham et al., 2010)—and more recently concerns of increased risk of bladder cancer have surfaced with pioglitazone (Lewis et al., 2011). TZDs increase water and salt retention by the kidneys, yet their use consistently lowers blood pressure, suggesting that the antihypertensive effects of tissue-specific PPAR γ activation in nonrenal tissues are potent (Ketsawatsomkron et al., 2010). While PPAR γ is well characterized as a master

regulator of adipogenesis, the direct actions of PPAR γ within the vasculature remain unclear. Given the concerns over TZD use, understanding the fundamental mechanisms of PPAR γ action in specific tissues is essential in order to identify therapeutic pathways that promote the beneficial actions of PPAR γ while minimizing detrimental actions. A new class of non-TZD ligands that prevent Cyclin-dependent kinase 5 (Cdk5)-dependent inhibition of PPAR γ and lack agonist activity may provide a similar level of glycemic control as TZD but without adverse effects on weight gain and fluid retention (Choi et al., 2011).

Individuals carrying mutations (i.e., P467L, V290M) and knockin mice with an equivalent mutation (P465L) in PPAR γ develop early-onset hypertension (Barroso et al., 1999; Tsai et al., 2004). Mutation of the ligand-binding domain of PPAR γ prevents ligand activation but retains its ability to bind DNA and complex with corepressors. Thus, these mutations confer dominant-negative (DN) activity, which alters PPAR γ -mediated transcription (Li and Leff, 2007). We demonstrated that smooth muscle cell-targeted expression of DN human PPAR γ in transgenic mice (termed S-P467L) caused elevated blood pressure and vascular dysfunction independent of changes in systemic metabolism (Halabi et al., 2008). This and other studies employing vascular-specific knockout of PPAR γ are consistent with the hypothesis that PPAR γ activation exerts its antihypertensive and vascular protective effects through actions in vascular endothelium and smooth muscle. Therefore, the aim of the current study is to elucidate the molecular mechanisms that underlie augmented contraction and impaired relaxation induced by expression of DN PPAR γ in smooth muscle.

RESULTS

DN PPAR γ -Induced Aortic Dysfunction Is Smooth-Muscle Specific

Smooth-muscle-specific expression of DN PPAR γ in S-P467L mice caused severely impaired relaxation to Ach and SNP (Figure 1A). Preincubation of aortic rings with L-NAME or removal of the endothelium completely abrogated Ach-induced relaxation in both S-P467L and NT but had no effect on SNP-induced relaxation (Figures 1A and S1A). The impairment in vasodilation is not due to increased superoxide because preincubation with Tempol, a scavenger of superoxide, did not alter the responses to either Ach or SNP (Figure S1B). The level of total and phospho-Ser¹¹⁷⁷ eNOS protein was similar in aortic tissue from NT and S-P467L mice (Figure S1C). ET-1 and 5-HT caused enhanced

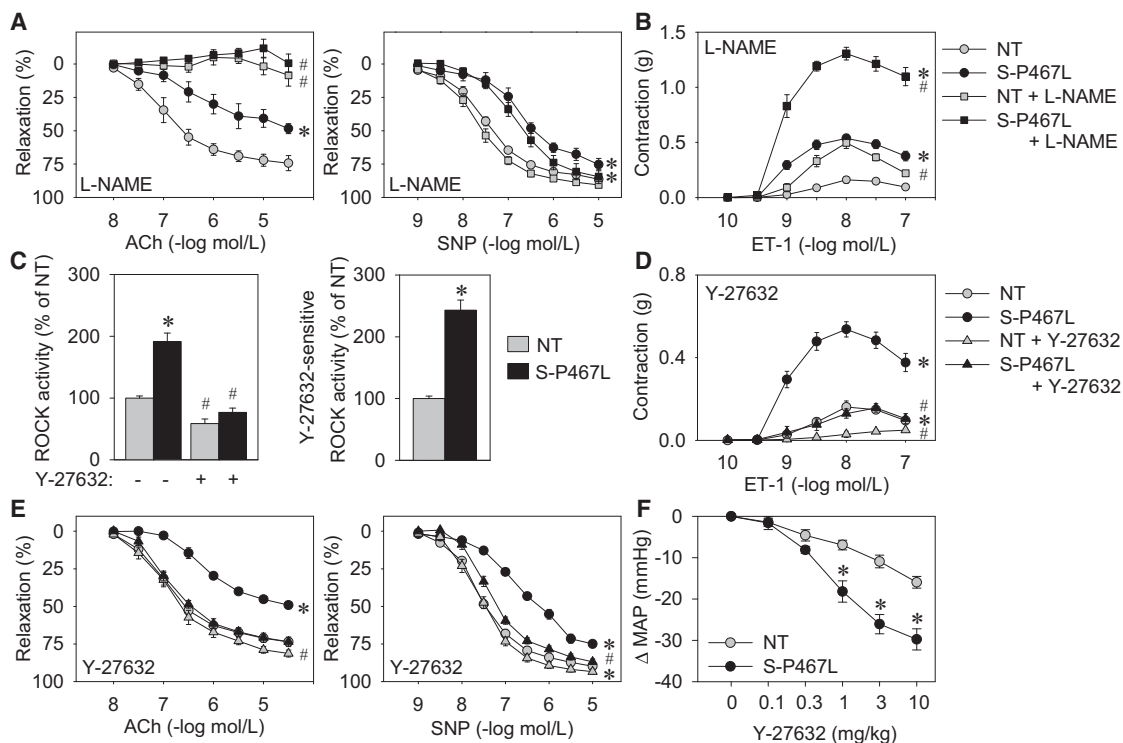


Figure 1. Interference with PPAR γ Causes Increased Rho-Kinase Activity

(A and B) Isometric tension was measured on aortic rings from S-P467L (black) and NT littermates (gray). Dose-dependent relaxation (following precontraction with prostaglandin $F_{2\alpha}$ [$PGF_{2\alpha}$]) in response to acetylcholine (ACh) and sodium nitroprusside (SNP) (A), contraction in response to endothelin 1 (ET-1) (B) was recorded in the absence or presence of NOS inhibitor (30 min preincubation; L-NAME, 100 μ mol/L) ($n = 6-12$).

(C) ELISA of phospho-Thr⁶⁹⁶ MYPT1 subunit of MLCP in the absence or presence of Rho-kinase inhibitor (Y-27632) using aortic tissue from NT and S-P467L mice ($n = 4-9$) and quantification of Y-27632-sensitive Rho-kinase activity.

(D and E) Effect of Rho-kinase inhibition (30 min preincubation; Y-27632, 1 μ mol/L) on contraction or relaxation of aortic rings from S-P467L and NT mice in response to ET-1 (D) or ACh and SNP (E) ($n = 9-12$).

(F) Direct blood pressure measurements were recorded in response to acute intravenous injection of the Rho-kinase inhibitor Y-27632 in anesthetized NT and S-P467L mice ($n = 6$). See also [Figures S1](#) and [S2](#). * $p < 0.05$ S-P467L versus NT. # $p < 0.05$ treated versus nontreated. Error bars represent SEM.

contraction of S-P467L aorta, which was augmented by either NOS inhibition or removal of the endothelium ([Figures 1B, S1D](#), and [S1E](#)). As above, pretreatment with Tempol had no effect on agonist-induced contraction ([Figure S1F](#)). These data suggest that NO production and bioavailability is unaltered and that aortic dysfunction in S-P467L mice is due to some other mechanism.

DN PPAR γ Increases Rho-Kinase Activity

We tested the hypothesis that the RhoA/Rho-kinase pathway is a downstream target of PPAR γ and that enhanced activation of Rho-kinase limits the effectiveness of NO/PKG signaling in S-P467L ([Chitale and Webb, 2002](#)). Basal Rho-kinase activity, measured by Thr⁶⁹⁶ phosphorylation of the myosin light chain phosphatase subunit MYPT1, was significantly increased in lysates from S-P467L aorta ([Figure 1C](#)). The increase in Rho-kinase activity was inhibited by Y-27632, a specific inhibitor of both Rho-kinase isoforms ROCK1 and ROCK2 ([Figure 1C](#)). Consistent with this, preincubation of aortic rings with Y-27632 blunted the hypercontractile responses from S-P467L aorta ([Figures 1D and S2A](#)), thus demonstrating that the enhanced contraction is Rho-kinase dependent. Importantly, preincuba-

tion with Y-27632 fully restored NO-mediated dilator responses in S-P467L aorta ([Figure 1E](#)). The improvement in ACh-induced relaxation after Rho-kinase blockade in S-P467L aorta was abrogated by NOS inhibition or endothelium removal indicating it is endothelium dependent ([Figure S2B](#)). This suggests that increased activity of the Rho-kinase pathway in smooth muscle is responsible for the DN PPAR γ -induced defect in NO-mediated relaxation. Likewise, intravenous injection of Y-27632 decreased mean arterial pressure by 30 ± 3 mmHg in S-P467L mice compared with 16 ± 1 mmHg ($p < 0.05$) in NT mice ([Figure 1F](#)), strongly suggesting the increase in arterial pressure caused by DN PPAR γ is Rho-kinase dependent.

Because Rho-kinase enhances the sensitivity of the contractile apparatus to Ca^{2+} , we tested if contraction to $CaCl_2$ was enhanced in ionomycin-permeabilized aortic rings. Aorta from S-P467L mice displayed increased contraction at lower concentrations of $CaCl_2$ compared to NT mice (S-P467L $EC_{50} = 4.3$ mmol/L versus NT $EC_{50} = 22.1$ mmol/L; $p < 0.05$) while maximum contraction was not different ([Figure S2C](#)). Similar to receptor-mediated agonists, Bay K8644 augmented contraction in S-P467L aorta that was both NO- and Rho-kinase dependent ([Figure S2D](#)). On the contrary, the PKC activator phorbol

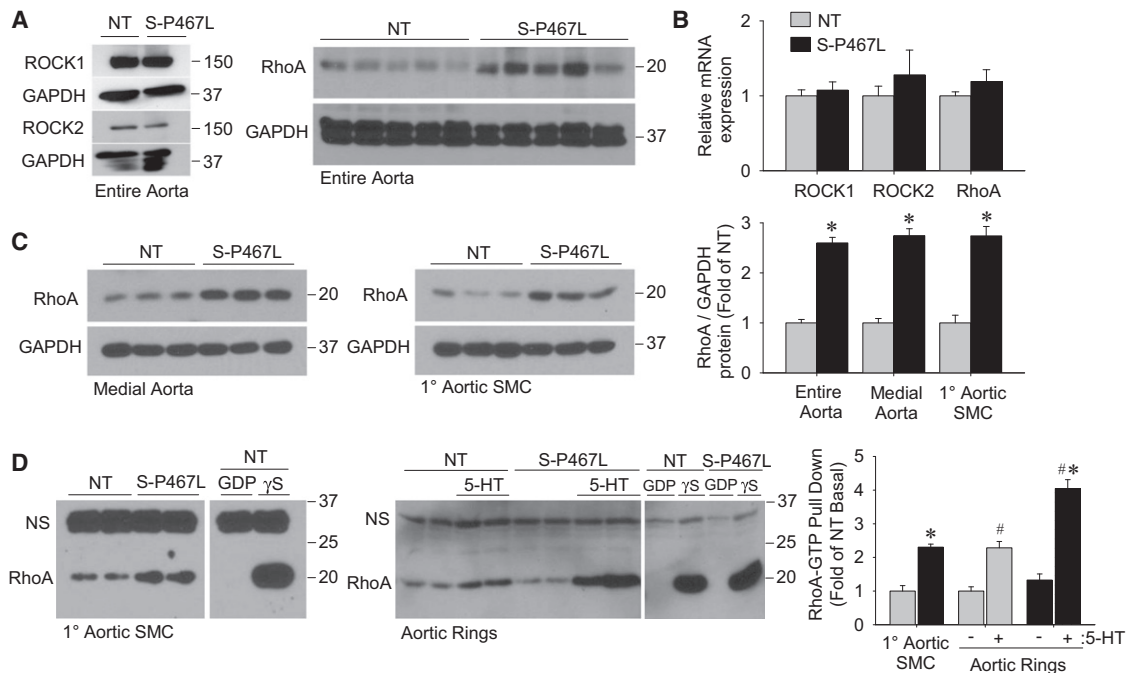


Figure 2. Increased RhoA Protein Expression and Activity

(A) Protein levels of ROCK1 and ROCK2 ($n = 4-5$) or RhoA ($n = 8$) in entire aorta of NT and S-P467L mice determined by western blot.

(B) Expression of ROCK1, ROCK2, and RhoA mRNA in aortic tissue determined by qPCR ($n = 10-11$).

(C) Total RhoA protein levels in medial aorta (dissected free of endothelium and adventitia) ($n = 8$) or 1° aortic smooth muscle cells (SMC) ($n = 6$) determined by western blot and quantification.

(D) Western blot of RhoA-GTP pull-down using nontreated 1° aortic SMC from NT and S-P467L mice ($n = 4$), and aortic rings equilibrated to 0.5 g tension plus or minus agonist-stimulation by 5-HT ($1 \mu\text{M/L}$, 5 min) ($n = 4$), and quantification. "NS" indicates nonspecific band. See also Figure S3. * $p < 0.05$ S-P467L versus NT. # $p < 0.05$ treated versus non-treated. Error bars represent SEM.

12,13-dibutyrate (PdBu) produced contraction that was similar between NT and S-P467L aorta and was not sensitive to L-NAME nor Y-27632 pre-incubation (Figure S2E).

DN PPAR γ Increases RhoA Protein and Activity

The mechanism for increased Rho-kinase activity was not due to a change in the protein levels of ROCK1 and ROCK2 (Figure 2A). On the contrary, the protein level of RhoA GTPase, which lies upstream and is a primary regulator of Rho-kinase, was increased in entire aorta from S-P467L mice under baseline conditions (Figure 2A). Despite this, the level of RhoA mRNA was unchanged in S-P467L aorta (Figure 2B). ROCK1 and ROCK2 mRNA levels were also unchanged (Figure 2B). The increase in RhoA is specific to the smooth muscle since RhoA protein was increased in medial aortic tissue (e.g., dissected free of endothelium and adventitia) and in primary aortic smooth muscle cells from S-P467L mice (Figure 2C). Increased total RhoA protein correlated with a greater basal level of active GTP-bound RhoA in primary aortic smooth muscle cells from S-P467L. Next, we determined the levels of GTP-bound RhoGTPases in basal or agonist-stimulated aortic rings equilibrated to resting tension (0.5 g). While the basal level of RhoA-GTP was not different between S-P467L and NT aortic rings at resting tension, 5-HT stimulation significantly increased RhoA-GTP to a greater extent in S-P467L aorta (Figure 2D). 5-HT produced the maximal difference in contraction between genotypes

(Figures S1D–S1F), and it induces GTP-bound RhoA (Sakurada et al., 2001). The level of GTP-bound Rac1 was unaffected by 5-HT stimulation and was not different between genotypes (Figure S3A). GTP-bound Cdc42 was not detected in the basal or stimulated state, but could be detected in control lysates incubated with GTP γ S (Figure S3B). Like RhoA, Rac1 and Cdc42 mRNA were unchanged in S-P467L aorta (Figure S3C). These data suggest that interference with PPAR γ in smooth muscle leads to enhanced Rho-kinase activity via increased expression and activity of its upstream regulator RhoA.

PPAR γ Regulates RhoBTB1 Expression

To gather insights into the molecular mechanism for the increase in RhoA/Rho-kinase activity, we performed gene-expression profiling on aorta from NT and S-P467L mice by employing both oligonucleotide and exon tiling arrays. Numerous PPAR γ target genes related to lipid metabolism were downregulated by DN PPAR γ in the aorta of S-P467L mice, consistent with PPAR γ 's role as a major regulator of adipogenesis. However, we focused on RhoBTB1, which was markedly decreased (Figure 3A), because it has previously been implicated to bind to Cullin-3 (Berthold et al., 2008), a cullin-RING E3 ubiquitin ligase complex scaffold protein that regulates the turnover of RhoA (Chen et al., 2009). RhoBTB1 mRNA and protein was decreased by approximately 72% and 34% as confirmed by real-time quantitative RT-PCR (Figure 3A) and western blot (Figure 3B),

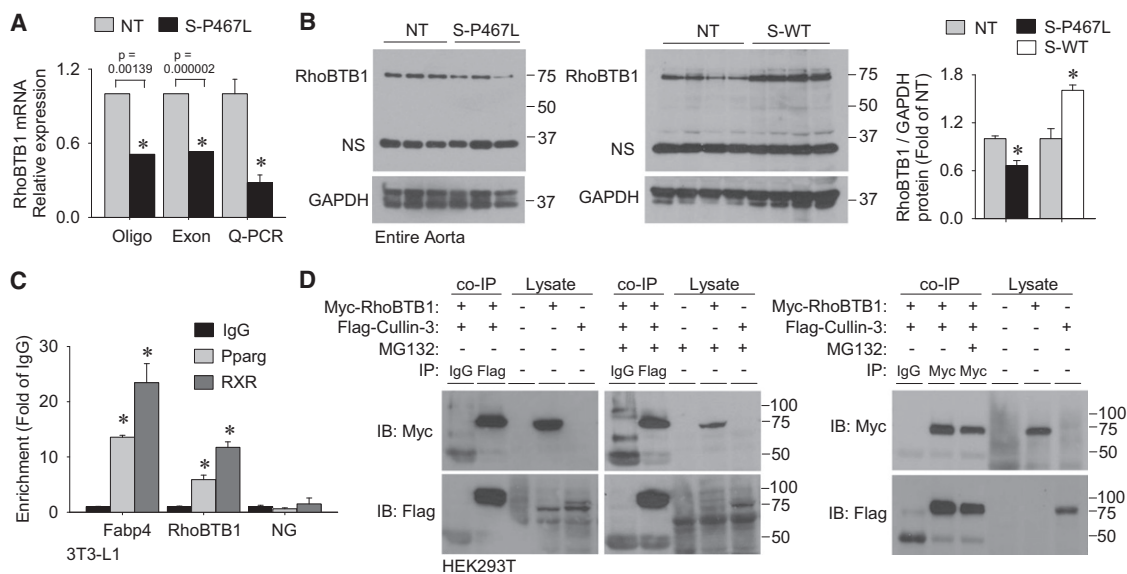


Figure 3. Identification of the Cullin-3-Interacting Protein RhoBTB1 as a PPAR γ Target Gene

(A) Relative mRNA expression of RhoBTB1 in aortic tissue from S-P467L mice relative to NT littermates in two independent microarray experiments using different array platforms and validation by Q-PCR (n = 10–11). Oligo array refers to the Affymetrix mouse genome 2.0 array (probe set 1429656_at, n = 2 for NT, and n = 3 for S-P467L), and exon array refers to the Affymetrix mouse exon 1.0 ST array (probe set 6768601, n = 5 for NT, and n = 7 for S-P467L).

(B) Western blot for RhoBTB1 protein expression in aorta from S-P467L and littermate NT mice (n = 8) or from S-WT and littermate NT mice (n = 4) and quantification. “NS” indicates nonspecific band.

(C) Validation of PPAR γ and RXR binding at FABP4 and RhoBTB1 PPREs by ChIP from duplicate experiments. Data are displayed as the fold enrichment relative to the IgG signal at the region of interest (*p < 0.05 Pparg or RXR versus IgG). NG represents a nonspecific region of the genome that does not contain any known PPRE sequences.

(D) Immunoprecipitation (IP) experiments using HEK293T cells transfected with plasmids expressing Myc-RhoBTB1, Flag-Cullin-3, or both. The antisera used for each IP is indicated. Lysates indicate samples not subjected to IP as controls for antisera specificity. Cells were treated with either MG132 (5 μ mol/L) or vehicle (DMSO). The left blots are representative of n = 4, and the right blot was representative of duplicate experiments. See also Figure S4. *p < 0.05 S-P467L or S-WT versus NT. Error bars represent SEM.

respectively. Evidence suggesting RhoBTB1 is a PPAR γ target gene was 3-fold. First, expression of RhoBTB1 protein was increased in aorta from transgenic mice overexpressing wild-type PPAR γ in smooth muscle (S-WT mice, Figure 3B). Second, PPAR γ and retinoid X receptor (RXR) binding sites were detected at the RhoBTB1 locus by genome-wide chromatin immunoprecipitation (ChIP, Figure S4A). Third, we validated PPAR γ binding at the RhoBTB1 locus using ChIP in differentiated 3T3-L1 cells (Figure 3C). Cullin-3 is known to interact with many BTB domain-containing proteins, including RhoBTBs, which may act as substrate adaptors and/or direct substrates of Cullin-3-dependent ubiquitylation (Berthold et al., 2008). Reciprocal IP, employing Flag-tagged Cullin-3 and Myc-tagged RhoBTB1 coexpressed in HEK293T cells, revealed direct interaction between RhoBTB1 and Cullin-3 that was not affected by proteasome inhibition (Figure 3D).

DN PPAR γ Impairs Cullin-3

The increase in total RhoA protein, but not mRNA, coupled with decreased RhoBTB1, suggested the hypothesis that DN PPAR γ in smooth muscle interferes with Cullin-3-mediated posttranslational regulation of RhoA. Cullin-3 protein and the ratio of neddylated Cullin-3 to unneddylated Cullin-3 was decreased in aorta from S-P467L mice (Figures 4A and 4B). The covalent attachment of Nedd8, called neddylation, is required for cullin-depen-

dent ubiquitin ligase activity (Wu et al., 2005). Total Nedd8 conjugates were detected only at the molecular weight corresponding to neddylated cullins (~90–95 kDa), whereas free monomeric Nedd8 (~8 kDa) was not detected. That there were no changes in the levels of Cullin-1, neddylated Cullin-1, or total Nedd8 conjugates in S-P467L aorta suggests the defect is specific to Cullin-3 (Figures 4A and 4B). Cullin-3 mRNA was not different between NT and S-P467L aorta (Figure 4B). The identity of the neddylated forms of cullins was confirmed by using the Nedd8-activating enzyme inhibitor MLN4924. Although there are no Cullin-specific (Cul1, 2, 3, 4A, 4B, 5, 7, 9) inhibitors, MLN4924 treatment effectively decreases the levels of neddylated cullins in human and mouse tissues (Soucy et al., 2009). Treatment of primary rat aortic smooth muscle cells with MLN4924 eliminated the presence of neddylated Cullin-3, neddylated Cullin-1, and total Nedd8 conjugates (Figures 4C and 4D). MLN4924 treatment also increased levels of RhoA, a Cullin-3 substrate (Figures 4C and 4D).

Cullin-3 Mediates RhoA Turnover in Smooth Muscle

To determine if the decrease in Cullin-3 is mechanistically related to the increase in RhoA protein in S-P467L aorta, we performed siRNA-mediated knockdown of Cullin-3 in primary rat aortic smooth muscle cells. There was no effect of negative control NC1 siRNA whereas two different siRNAs directed against

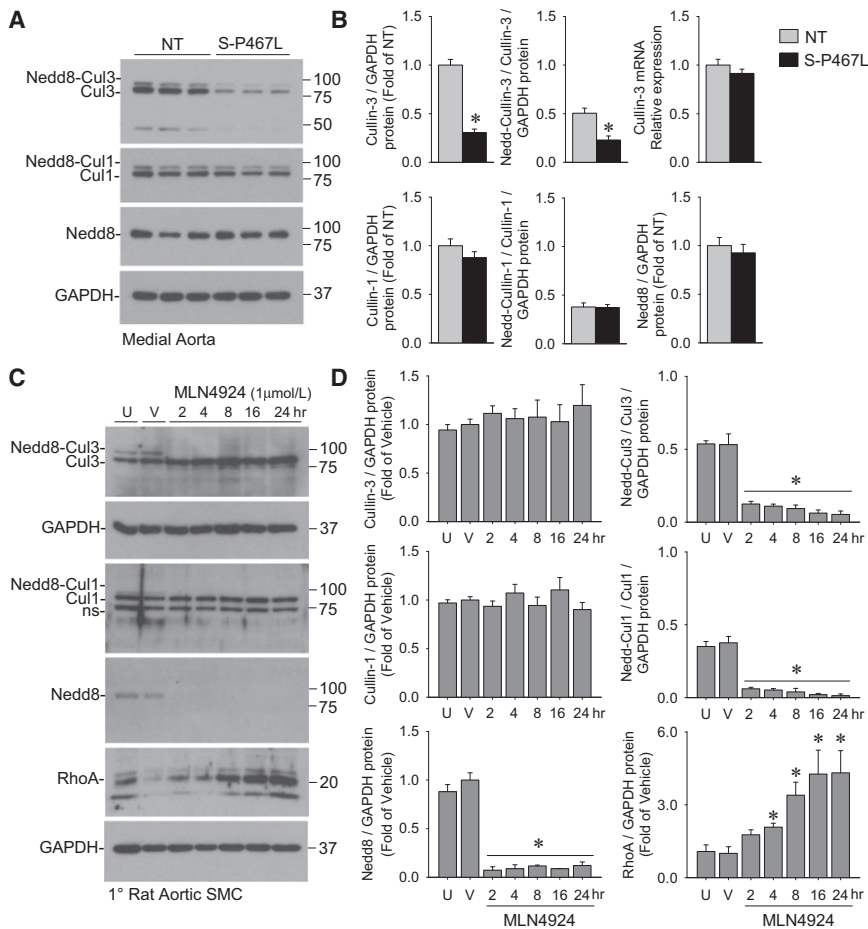


Figure 4. Interference with PPAR γ Causes Decreased Cullin-3 and Nedd8-Cullin-3

(A and B) Protein levels of Cullin-3 and neddylated Cullin-3, Cullin-1 and neddylated Cullin-1, and total Nedd8 conjugates (Nedd8) in medial aortic tissue from S-P467L and NT mice determined by western blot ($n = 8-9$) and their quantification. Neddylation is the covalent attachment of Nedd8 onto a target protein. The lower molecular weight band of the doublet (~ 85 kDa) is unneddylated Cullin-3 or Cullin-1 (denoted as Cul3 or Cul1) whereas the higher band (~ 95 kDa) is the neddylated Cullin-3 or Cullin-1 product (denoted as Nedd8-Cul3 or Nedd8-Cul1). Cullin-3 mRNA expression determined by qPCR ($n = 10$). See also Table S1. * $p < 0.05$ S-P467L versus NT. Error bars represent SEM.

(C and D) Primary rat aortic smooth muscle cells treated with the Nedd8-activating enzyme inhibitor MLN4924 (1 μ mol/L) or DMSO over a time course of 24 hr. Protein lysates were assessed for Cullin-3, Cullin-1, Nedd8, and RhoA by western blot and quantified ($n = 4$). U, untreated; V, vehicle (DMSO, 24 hr). "NS" indicates nonspecific band. * $p < 0.05$ MLN4924 treated versus vehicle treated. Error bars represent SEM.

Cullin-3 decreased its expression by nearly 85% (Figure 5A). Loss of Cullin-3 expression caused a robust increase in RhoA and interestingly also increased levels of RhoBTB1 (Figure 5B). This suggests that RhoBTB1 is a substrate of Cullin-3-mediated degradation and explains why the level of RhoBTB1 protein was only modestly decreased in S-P467L aorta. Cyclin E, another substrate of Cullin-3-mediated degradation (Kossatz et al., 2010), was also increased by siRNA-mediated Cullin-3 knockdown in cells (Figure 5B) and in S-P467L aorta (data not shown). siRNA-mediated knockdown of RhoBTB1 in primary rat aortic smooth muscle cells caused a modest yet significant decrease in Cullin-3 and neddylated Cullin-3 protein and a trend toward increased RhoA, but this was not as dramatic as Cullin-3 knockdown (Figure S4B). Interestingly, analysis of both oligonucleotide and exon microarrays revealed no changes in the level of mRNA-encoding proteins involved in neddylation and BTB-domain containing proteins other than RhoBTB1 (Table S1).

Cullin-RING Ligase Activity Regulates Vascular Function and Blood Pressure

We next tested the hypothesis that inhibition of cullin-RING ligase activity leads to enhanced contraction. We established above that RhoA protein levels were robustly upregulated by 16 hr after treatment with MLN4924 (Figures 4C and 4D). Therefore, aortic rings from NT mice were first incubated for 16 hr

ex vivo with MLN4924 or vehicle and then isometric tension studies were performed in the presence of the inhibitor. MLN4924 enhanced agonist-induced contraction to ET-1, 5-HT, and PE in NT aorta, despite blunting receptor-independent contraction to KCl (Figures 6A–6D). Preincubation with the Rho-kinase inhibitor Y-27632 was effective at blocking the MLN4924-dependent enhanced contractile responses (Figures 6A–6D). Acute treatment of the vessels with MLN4924 for only 1 hr was not sufficient to alter reactivity, whereas long-term treatment of aortic rings from NT mice ex vivo with MLN4924 for 6 days produced the same results as 16 hr (data not shown). We next tested if the enhanced agonist-induced contraction in S-P467L mice was occurring through this mechanism. Consistent with a loss of Cullin-3 mediated activity, the effect of MLN4924 was markedly blunted in S-P467L aorta (Figures 6E–6H). Together these data show that inhibition of cullin-RING ligase activity causes striking alterations in vascular contractility, likely in part through loss of Cullin-3-mediated regulation of RhoA/Rho-kinase signaling.

Finally, to provide an in vivo correlate to the vascular function experiments, we measured arterial pressure by radiotelemetry in conscious NT and S-P467L mice in response to MLN4924 using a previously established dose regimen (Soucy et al., 2009; Smith et al., 2012). Compared to baseline, continuous administration of MLN4924 (subcutaneous injections; 30 mg/kg TID, 2.5 days) caused a significant increase in daytime arterial pressure in normal NT mice (Figures 7A and S5). Baseline arterial pressure was significantly higher in S-P467L mice, but MLN4924 treatment did not raise arterial pressure further. MLN4924 treatment caused a marked decrease in spontaneous activity during the nighttime hours (Figure 7B) that was equivalent in both NT and

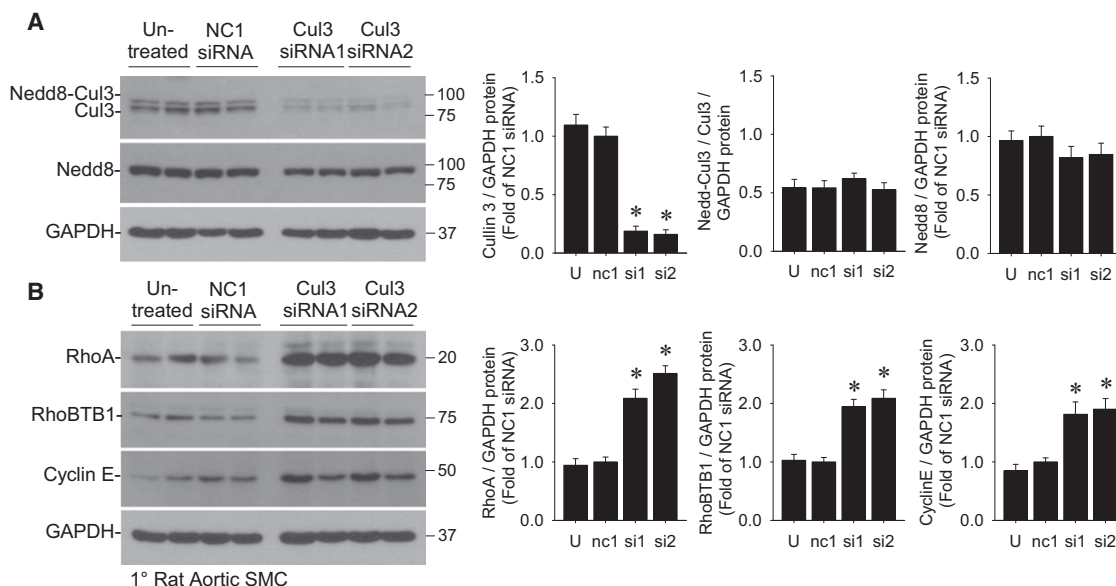


Figure 5. siRNA knockdown of Cullin-3 Increases RhoA in Rat Aortic Smooth Muscle Cells

(A and B) 1° rat aortic SMC treated with negative control siRNA (NC1) or two different siRNAs targeting Cullin-3 were assessed for protein levels of Cullin-3 and total Nedd8-conjugates (Nedd8) (A) and Cullin-3 substrates RhoA, RhoBTB1, and Cyclin E (B) by western blot ($n = 6$). U, untreated; nc1, negative control siRNA; si1, Cullin-3 siRNA #1; si2, Cullin-3 siRNA #2. * $p < 0.05$ Cullin-3 siRNA treated versus NC1 siRNA treated. Error bars represent SEM.

S-P467L mice. The decrease in activity correlated to a loss of normal diurnal variation in arterial pressure and heart rate that was also independent of genotype (Figures 7A–7C). MLN4924 administration significantly reduced the level of neddylated Cullin-3 and total Nedd8-conjugates and increased RhoA protein in aortic tissue from NT mice (Figure 7D). These data suggest that cullin-RING ligase activity may be important for blood-pressure regulation and are consistent with the hypothesis that loss of Cullin-3 in the vasculature contributes to hypertension. Indeed, mutations in Cullin-3 cause severe early-onset hypertension (Boyden et al., 2012), although the tissue-specific actions of Cullin-3 remain unclear. The hypertension in patients harboring Cullin-3 mutations was associated with changes in ion homeostasis, namely potassium and bicarbonate balance (Boyden et al., 2012). Blood electrolytes, except for decreased blood urea nitrogen (BUN), were normal in untreated S-P467L mice compared to NT mice (Table S2). Systemic MLN4924 treatment caused mild hyperkalemia, decreased hematocrit, and increased BUN that was also not specific to genotype (Table S2).

DISCUSSION

PPAR γ within the vascular wall has a pivotal role in protecting against vascular dysfunction or abnormal vascular growth and remodeling during hypertension (Diep et al., 2002); however, the mechanisms behind such improvements remain unclear. PPAR/RXR heterodimers, bound to corepressors, actively silence transcription at PPAR response elements (PPREs). Ligand activation stimulates coactivators to replace corepressors and allow transcription of target genes (Gronemeyer et al., 2004). Interestingly, genetic deletion of PPAR γ causes induction of some genes due to loss of PPAR-dependent active repres-

sion, which may mimic PPAR γ activation (Chang et al., 2009; Sigmund, 2010). Our approach was guided by the identification of bona fide mutations in the PPAR γ ligand-binding domain from patients who developed early-onset hypertension and insulin resistance (Barroso et al., 1999; Savage et al., 2003). Mice homozygous for the equivalent PPAR γ P465L knockin mutation die in utero, confirming that the DN mutation is detrimental to receptor function (Tsai et al., 2004). PPAR γ P465L heterozygous (P465L/+) mice develop hypertension and features of metabolic syndrome (Tsai et al., 2004). Global gene-expression analysis validated that PPAR γ target genes induced by agonist treatment were also repressed by DN PPAR γ in vascular tissue (Keen et al., 2010; Keen et al., 2004). Herein, we combined physiological, biochemical, and bioinformatic approaches to identify PPAR γ target genes that regulate vascular function and arterial pressure.

The mechanisms contributing to vascular dysfunction induced by smooth muscle-specific expression of DN PPAR γ are as follows: (1) endothelium-dependent production of NO is preserved; (2) enhanced Rho-kinase activity is responsible for increased agonist-induced contraction, impaired NO-mediated relaxation, and increased arterial pressure in S-P467L mice; (3) increased RhoA protein and RhoA-GTP upstream of Rho-kinase results from impaired Cullin-3-mediated RhoA turnover in vascular muscle; (4) RhoBTB1, which interacts with Cullin-3, is a PPAR γ target gene and is downregulated by DN PPAR γ in vascular muscle; (5) pharmacological inhibition of cullin-RING ligase activity in aortic segments from wild-type mice causes augmented agonist-induced contraction that is Rho-kinase dependent; and (6) inhibition of cullin-RING ligase activity in wild-type mice in vivo causes increased daytime arterial pressure.

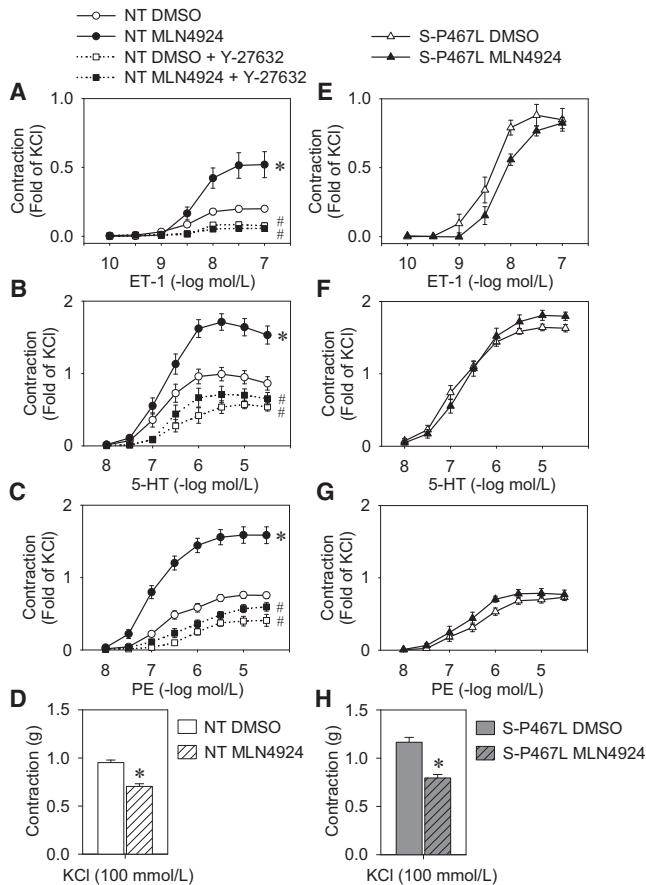


Figure 6. Inhibition of Total Cullin Activity Enhances Agonist-Mediated Contraction

(A–H) Isometric tension data on aortic rings from NT mice (A–D; $n = 10$) or S-P467L mice (E–H; $n = 6$) incubated in DMEM/F12 (37°C, 5% CO₂) with DMSO or MLN4924 (1 μ M/L) for 16 hr. DMSO and MLN4924 were continuously applied throughout the tension experiments. Contractile responses were recorded to ET-1 (A and E), 5-HT (B and F), phenylephrine (PE) (C and G), or KCl (D and H). Aortic rings from NT mice (A–D) were further studied in the absence or presence of Rho-kinase inhibitor (30 min preincubation; Y-27632, 1 μ M/L) ($n = 6$). Data are normalized to maximum contraction (fold of 100 mmol/L KCl). * $p < 0.05$ MLN4924 treated versus vehicle treated. # $p < 0.05$ Y-27632 treated versus MLN4924- or vehicle treated alone. Error bars represent SEM.

Increased RhoA/Rho-kinase activity is implicated in the pathogenesis of many cardiovascular diseases, although the underlying causes are not well understood (Budzyn et al., 2006). Basal release of NO from the endothelium prevents activation of RhoA/Rho-kinase in smooth muscle through PKG-dependent inhibitory phosphorylation of RhoA (Sauzeau et al., 2000). Excessive reactive oxygen species (ROS; e.g., superoxide) inactivate NO, leading to a reduction of NO bioavailability and increased vascular tone, at least in part through elevated RhoA/Rho-kinase activity (Jin et al., 2006). However, our results suggest that increased activation of Rho-kinase in S-P467L aorta is neither a result of oxidative stress nor decreased bioavailability of endothelium-derived NO but is most likely due to a primary defect in the smooth muscle. This is consistent with the genetic lesion being targeted to the smooth muscle.

We next sought to determine the primary PPAR γ -dependent effect on regulators upstream of Rho-kinase. The dual regulation of MLC phosphorylation through Ca²⁺-dependent activation of MLCK and RhoA/Rho-kinase-dependent inhibition of MLCP is attributed to the coupling of G protein-coupled receptors (GPCRs) to G $\alpha_{q/11}$ and G $\alpha_{12/13}$, respectively (Maguire and Davenport, 2005; Wirth et al., 2008). Stimulation of aortic smooth muscle cells with agonists of GPCRs including ET-1, 5-HT, and PE, in addition to high KCl, enhances RhoA-GTP, which activates Rho-kinase (Sakurada et al., 2001, 2003; Momotani et al., 2011). We provide clear evidence of increased RhoA protein, RhoA-GTP, and Rho-kinase activity in S-P467L aorta. Thus, PPAR γ -dependent regulation of the RhoA/Rho-kinase and NO/PKG-dependent signaling in smooth muscle is key in shifting the balance between vasoconstrictor and vasodilator responses. The PPAR γ agonist pioglitazone was shown to lower systolic blood pressure and suppress RhoA/Rho-kinase activity in aortic tissue of spontaneously hypertensive rats (SHR) (Wakino et al., 2004). Similarly, upregulation of RhoA in Dahl salt-sensitive hypertensive rats was decreased by telmisartan, a dual ARB/PPAR γ agonist (Kobayashi et al., 2008). Whereas reports have demonstrated that activation of PPAR γ with TZDs suppresses RhoA/Rho-kinase signaling in microvascular endothelial cells (Ramirez et al., 2008), monocytes (Toriumi et al., 2003), and neurons (Dill et al., 2010), the mechanisms are not established.

Expression of DN PPAR γ in smooth muscle of S-P467L mice caused increased RhoA protein level in the aorta; however, this was not associated with a change in RhoA mRNA, suggesting a potential alteration in posttranslational regulation of RhoA. This was supported by gene-expression profiling, revealing a marked decrease in RhoBTB1, an atypical Rho GTPase that lacks GTPase activity. RhoBTB1 binds to the N-terminal region of Cullin-3 through its BTB domain (Berthold et al., 2008). RhoA is a substrate targeted by the Cullin-3 E3 RING ubiquitin ligase complex for ubiquitylation and subsequent degradation in the proteasome (Chen et al., 2009). Silencing of human Cullin-3 caused a loss of RhoA ubiquitylation and accumulation of RhoA protein and RhoA-GTP—and induced actin stress fiber formation that was RhoA-dependent in HeLa cells (Chen et al., 2009). We found decreased levels of Cullin-3 and neddylated Cullin-3 in aorta from S-P467L mice. The decreased ratio of Nedd8-conjugated Cullin-3 to unneddylated Cullin-3 is important because the neddylation of cullins is required for cullin-RING ubiquitin ligase activity (Petroski and Deshaies, 2005). Our data support that loss of Cullin-3 in primary rat aortic smooth muscle cells causes accumulation of its substrates, including RhoA and Cyclin E, and raised the possibility that inhibition of Cullin-3 activity may regulate vascular reactivity. To test this, we used MLN4924, an inhibitor of Nedd8-activating enzyme, which catalyzes Nedd8 conjugation onto cullins (Soucy et al., 2009). MLN4924 treatment blocked neddylation of Cullin-3 and Cullin-1 and completely blocked the formation of total Nedd8-conjugates in primary rat aortic smooth muscle cells. This suggests a remarkable specificity of Nedd8 conjugation onto cullins in vascular tissue. Treatment of aorta from wild-type (NT control) mice with MLN4924 caused increased agonist-induced contraction that, despite the broad effects of cullin inhibition, was Rho-kinase dependent. This is consistent with studies

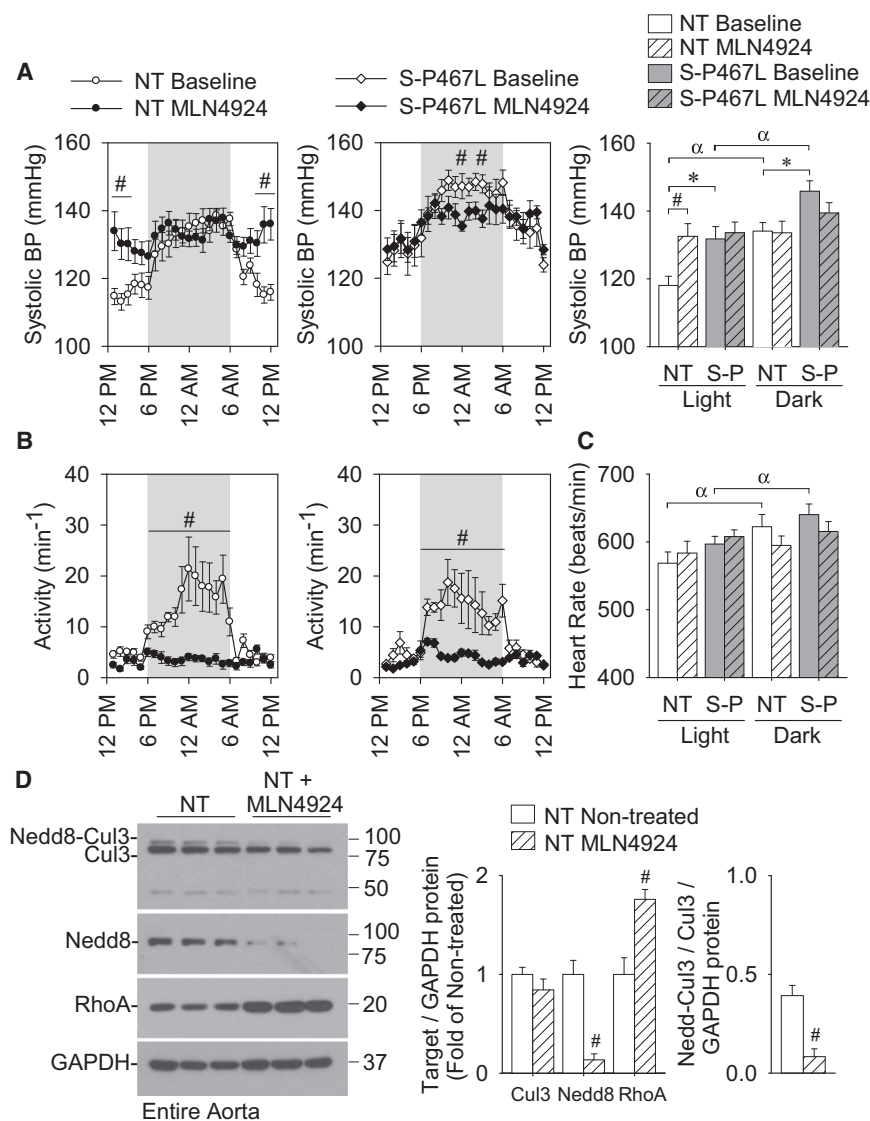


Figure 7. Inhibition of Total Cullin Activity Increases Daytime Blood Pressure in Normal Mice

(A–C) Systolic blood pressure (A), spontaneous activity (B), and heart rate (C) recorded continuously throughout the light:dark cycle by radiotelemetry in NT ($n = 9$) and S-P467L mice ($n = 7$). Baseline measurements were taken for 7 days. At the beginning of the light phase, mice began continuous dosing with MLN4924 (subcutaneous injections; 30 mg/kg TID). Data represent the average of the baseline measurements (7 days) and the average of MLN4924 treatment over 2.5 days (60 hr). See also Figure S5 and Table S2. # $p < 0.05$ MLN4924 treatment versus baseline. * $p < 0.05$ S-P467L versus NT. α $p < 0.05$ dark cycle versus light cycle. Error bars represent SEM.

regulated by DN PPAR γ in S-P467L aorta was instrumental in discovering the accompanying decrease in Cullin-3, and suggests that RhoBTB1 may regulate Cullin-3 level or activity. It has been hypothesized that RhoBTBs may regulate the targeting of specific Cullin-3 substrates, including RhoA. This is based on the observation that RhoBTB1 and RhoBTB2 are candidate tumor suppressors, and their loss correlates with increased RhoA protein in breast and head and neck cancers (Beder et al., 2006; Nethe and Hordijk, 2010). Our results from siRNA knockdown of RhoBTB1 suggest that its down-regulation partially explains decreased Cullin-3 levels, indicating perhaps some other PPAR γ -dependent or PPAR γ -independent effects are involved in this mechanism. Some candidates are BTB domain-containing RhoA-binding proteins (BACURDs), which were recently identified to be adaptors for Cullin-3 mediated RhoA ubiquitylation (Chen et al., 2009). Expression of BACURDs, at least at the mRNA level, did not appear to be regulated by PPAR γ in vascular muscle on the basis of our microarray data (Table S1). Identifying which adaptor proteins control substrate specificity for Cullin-3 remains under active investigation, and new substrates for Cullin-3:RhoBTB-domain-mediated ubiquitylation continue to be uncovered (Schenkova et al., 2012). Additional investigation will be required to determine the full range of substrates the RhoBTB family of proteins anchor to Cullin-3.

reporting that MLN4924 treatment of HEK293 cells caused accumulation of RhoA protein and altered cell morphology that was completely reversed by siRNA-knockdown of RhoA (Leck et al., 2010). Our in vivo studies revealed that systemic administration of NT mice with MLN4924 caused a significant increase in arterial pressure, at least during the light phase, whereas its administration to S-P467L mice did not. This supports our conclusion that the impairment of Cullin-3-mediated regulation of RhoA in the vasculature contributes to hypertension. Systemic MLN4924 treatment also had nonspecific effects on the normal diurnal variation in arterial pressure, heart rate, and activity. Consequently, use of gene-targeted mice would better clarify the actions of specific cullins in the blood vessel.

Cullin-based E3 ubiquitin ligases are dimeric, and Cullin-3/substrate homooligomers are dependent on substrate dimer formation (Chew et al., 2007). Cullin-3 is the only cullin-RING ligase member reported to bind to BTB domain-containing proteins for its substrate specificity (Petroski and Deshaies, 2005). The finding that RhoBTB1 is a PPAR γ target gene down-

regulated by DN PPAR γ in S-P467L aorta was instrumental in discovering the accompanying decrease in Cullin-3, and suggests that RhoBTB1 may regulate Cullin-3 level or activity. It has been hypothesized that RhoBTBs may regulate the targeting of specific Cullin-3 substrates, including RhoA. This is based on the observation that RhoBTB1 and RhoBTB2 are candidate tumor suppressors, and their loss correlates with increased RhoA protein in breast and head and neck cancers (Beder et al., 2006; Nethe and Hordijk, 2010). Our results from siRNA knockdown of RhoBTB1 suggest that its down-regulation partially explains decreased Cullin-3 levels, indicating perhaps some other PPAR γ -dependent or PPAR γ -independent effects are involved in this mechanism. Some candidates are BTB domain-containing RhoA-binding proteins (BACURDs), which were recently identified to be adaptors for Cullin-3 mediated RhoA ubiquitylation (Chen et al., 2009). Expression of BACURDs, at least at the mRNA level, did not appear to be regulated by PPAR γ in vascular muscle on the basis of our microarray data (Table S1). Identifying which adaptor proteins control substrate specificity for Cullin-3 remains under active investigation, and new substrates for Cullin-3:RhoBTB-domain-mediated ubiquitylation continue to be uncovered (Schenkova et al., 2012). Additional investigation will be required to determine the full range of substrates the RhoBTB family of proteins anchor to Cullin-3.

A role for Cullin-3 in hypertension has recently been reported (Boyden et al., 2012). Remarkably, greater than 90% of patients with dominant mutations in *cullin-3* developed hypertension before the age of 18, as did about 15% of patients with mutations in *KLHL3*, a Cullin-3 substrate adaptor. The mutations were discovered from a group of patients with pseudohypoaldosteronism type II (PHAII), a syndrome characterized by hypertension, hyperkalemia, and metabolic acidosis. Our data indicate that

hypertension caused by smooth muscle-specific expression of DN PPAR γ in S-P467L transgenic mice is not related to changes in electrolyte balance. Patients with DN mutations in PPAR γ also develop early-onset hypertension but have normal serum potassium levels (Savage et al., 2003). In PHAI, *cullin-3* mutations may not only perturb renal ion transport as demonstrated for mutations in one of its partners *KLHL3* (Louis-Dit-Picard et al., 2012), but perhaps may also provoke a severe and early-onset hypertension (Boyden et al., 2012) by increasing vascular contraction and impairing the response to NO. Together these findings lead to the hypothesis that hypertension caused by *cullin-3* mutations may be due in part to impaired Cullin-3-mediated regulation of RhoA/Rho-kinase in vascular smooth muscle. We conclude that interference with PPAR γ signaling, either through mutation or perhaps through inhibitory modification (i.e., Cdk5-mediated phosphorylation at Serine 273) (Choi et al., 2011), causes a decrease in Cullin-3 activity associated with loss of the Cullin-3-interacting protein RhoBTB1. Impaired Cullin-3-dependent degradation of RhoA results in enhanced RhoA/Rho-kinase signaling and causes an imbalance in responsiveness to vasodilator and vasoconstrictor signals leading to hypertension. Hence, under normal conditions PPAR γ facilitates tight regulation of RhoA/Rho-kinase activity via Cullin-3-mediated turnover of RhoA. Future studies are needed to discern the extent that aberrant regulation of Cullin-3 activity or expression in the vasculature and other tissues contributes to hypertension and cardiovascular disease.

EXPERIMENTAL PROCEDURES

Transgenic Mouse Model

S-P467L transgenic mice were described previously and were backcross bred to C57BL/6J for at least ten generations (Halabi et al., 2008). All mice were fed standard mouse chow (7013, Teklad Premier Laboratory Diets) and water ad libitum. Care of the mice used in the experiments met the standards set forth by the National Institutes of Health (NIH) guidelines for the care and use of experimental animals. All procedures were approved by the University of Iowa Institutional Animal Care and Use Committee.

Wire Myograph Preparation

Isometric tension was measured on aorta from mice 5–6 months of age as reported (Halabi et al., 2008; Beyer et al., 2008). Mice were given a lethal dose of pentobarbital (50 mg/mouse IP). Thoracic aorta was removed and placed in oxygenated Krebs buffer, dissected free of adventitial fat, and cut into 4 segments 4–5 mm in length. Where necessary the endothelial cell layer was removed by rubbing the lumen with a trimmed wooden toothpick. Aortic rings were suspended in organ baths containing 20 ml Krebs buffer (maintained at 37°C and 95% O₂/5% CO₂) and connected to a force transducer to measure isometric tension; resting tension was set to 0.5 g, and the aortic rings were equilibrated for 45 min. Contraction was recorded for ET-1 (0.1–100 nmol/L), 5-HT (0.01–30 μ mol/L), PE (0.01–30 μ mol/L), PGF_{2 α} (0.1–30 μ mol/L), and KCl (10–100 mmol/L). Relaxation was recorded for Ach (0.01–30 μ mol/L), SNP (1–10,000 nmol/L) after initial submaximal precontraction (40%–50% of max) with PGF_{2 α} . In some experiments, two aortic rings were preincubated (30 min) with the drug, while two other segments were untreated. These include 100 μ mol/L L-NAME, 1 μ mol/L Y-27632, and 10 μ mol/L Tempol. For studies on Ca²⁺ sensitivity, aortic rings were equilibrated, then switched to Ca²⁺-free Krebs buffer with three washes over a period of 15 min. Vessels were then treated with 3 μ mol/L ionomycin (in DMSO) for 30 min. Contractile responses were recorded for CaCl₂ (0.01 μ mol/L–100 mmol/L in Ca²⁺-free Krebs). For experiments using MLN4924, aortic rings were incubated in DMEM/F12 (16 hr or 6 day; 37°C,

5% CO₂) with either DMSO or 1 μ mol/L MLN4924 (in DMSO) prior to preparation onto wire myograph. DMSO or 1 μ mol/L MLN4924 was continuously applied to organ baths after each wash during equilibration and throughout the length of the tension studies. Data were collected with PowerLab/8SP and analyzed with Chart 5 software (AD Instruments).

Blood Pressure Analysis

First, S-P467L and NT mice 5–6 months of age were anesthetized with pentobarbital (60 mg/kg intraperitoneally), and cannulation of the carotid artery and jugular vein with 0.040 O.D. sterile tubing was performed. Acute blood-pressure responses were measured during anesthesia. Depressor effects were measured in response to Y-27632 (0.1–10 mg/kg through the venous catheter). Data were collected with PowerLab/8SP and analyzed with Chart 5 software (AD Instruments). Second, blood pressure was measured by radiotelemetry as described (Beyer et al., 2008; Halabi et al., 2008). After 7 days of recovery, arterial pressure, heart rate, and activity were continuously recorded (sampling every 5 min for 20 s intervals). Baseline blood pressure was measured for 7 days. At the beginning of the light phase, mice began continuous treatment with MLN4924 (in 10% hydroxy-propyl- β -cyclodextrin) by subcutaneous injections (30 mg/kg TID), and blood pressure was recorded and analyzed over the next 60 hr (2.5 days). Data were collected and stored using Dataquest ART. Mice were sacrificed at 1 hr following the final injection at 72 hr (3 days). Cheek puncture blood samples were collected and analyzed using i-STAT system CG4+ and CHEM8+ cartridges. Aortic tissue was harvested for protein analyses.

Cell Culture, siRNA Gene Silencing, and Western Blotting

Primary smooth muscle cells were isolated from thoracic aorta of S-P467L or NT mice. They were dissected free of perivascular fat; cut into small pieces; and dissociated with collagenase, elastase, soybean trypsin inhibitor, and bovine serum albumin type I at 37°C for 30 min. The suspension was centrifuged at 2000 rpm for 5 min, the pellet was resuspended, and cells were plated and maintained in DMEM with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C and 5% CO₂. The medium was replaced every 48 hr. Once confluent, the cells were passaged and verified as smooth muscle by staining with α -actin and calponin. Subconfluent smooth muscle cells at passages 2–4 were used for experiments. Primary culture of aortic smooth muscle cells from Sprague Dawley rat was a gift from Amy Banes-Berceli (Oakland University, MI). Rat aortic smooth muscle cells were maintained in low glucose (1 g/L) DMEM with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin and kept at 37°C and 5% CO₂. Cells were cultured 1 day prior to transfection with 20 nmol/L negative control NC1 siRNA or siRNA duplexes targeted to rat Cullin-3 or RhoBTB1 (Integrated DNA Technologies; Coralville, IA) using HiPerfect (QIAGEN) using the manufacturer's protocol. Cells were harvested 72 hr later and total protein lysates were stored at –80°C. Rat aortic smooth muscle cells were treated with MLN4924 (1 μ mol/L) for 2, 4, 8, 16, and 24 hr or vehicle control (DMSO) for 24 hr and harvested for total protein lysates. Experimental procedures for tissue preparation, transfections, immunoprecipitation, western blotting, and the antibodies used are described in detail in the [Supplemental Experimental Procedures](#).

Rho-Kinase and Rho GTPase Pull-Down Assay

Rho-kinase-dependent phosphorylation of its substrate MYPT1 was measured in aorta and primary aortic smooth muscle cells by ELISA, and Rho GTPase activity was determined by pull-down for the active GTP-bound forms of RhoA, Rac1, or Cdc42. The assays are described in detail in the [Supplemental Experimental Procedures](#).

Real-Time RT-PCR, Microarray Analysis, and Chromatin Immunoprecipitation

Real-time PCR (qPCR) analysis was performed using TaqMan Gene Expression Assays. Two independent microarray experiments (series accession: GSE37196) were performed using different array platforms: the Affymetrix mouse genome 2.0 array and the Affymetrix mouse exon 1.0 ST array. Chromatin immunoprecipitation was performed on differentiated 3T3-L1 cells. The experimental procedures for these analyses are described in detail in the [Supplemental Experimental Procedures](#).

Drugs and Reagents

Ach, SNP, KCl, 5-HT, PE, and N ω -Nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma; PGF $_{2\alpha}$ from Pfizer; and Y-27632 from Calbiochem; all of these reagents were dissolved in saline. ET-1 was obtained from Peninsula Laboratories Inc., and dissolved in water. MLN4924 (Active-Biochem), ionomycin (Calbiochem), Bay K8644 (Sigma), and Phorbol 12,13-dibutyrate (Sigma) were dissolved in DMSO.

Statistical Analyses

All data are expressed as mean \pm SEM. Data were analyzed with 1- or 2-way analysis of variance (ANOVA, repeated measures when appropriate) using Tukey or Bonferroni post hoc tests. Data sets failing normality or equal variance assumptions, or with small n ($n < 5$), were analyzed using nonparametric comparisons (Friedman's ANOVA or Mann-Whitney U Test). $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at <http://dx.doi.org/10.1016/j.cmet.2012.08.011>.

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